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Coordinate Intracellular Expression of *Salmonella* Genes Induced during Infection

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Salmonella typhimurium **in vivo-induced (*ivi*) genes were grouped by their coordinate behavior in response to a wide variety of environmental and genetic signals, including pH, Mg^{2+}, Fe^{2+}, and PhoPQ. All of the seven *ivi* fusions that are induced by both low pH and low Mg^{2+} (e.g., *iviVI-A*) are activated by the PhoPQ regulatory system. Iron-responsive *ivi* fusions include those induced under iron limitation (e.g., *entF*) as well as one induced by iron excess but only in the absence of PhoP (*pdu*). Intracellular expression studies showed that each of the pH- and Mg^{2+}-responsive fusions is induced upon entry into and growth within three distinct mammalian cell lines: RAW 264.7 murine macrophages and two cultured human epithelial cell lines: HEp-2 and Henle-407. Each *ivi* fusion has a characteristic level of induction consistent within all three cell types, suggesting that this class of coordinately expressed *ivi* genes responds to general intracellular signals that are present both in initial and in progressive stages of infection and may reflect their responses to similar vacuolar microenvironments in these cell types. Investigation of *ivi* expression patterns reveals not only the inherent versatility of pathogens to express a given gene(s) at various host sites but also the ability to modify their expression within the context of different animal hosts, tissues, cell types, or subcellular compartments.**

We have implemented a genetic system, termed IVET (in vivo expression technology), to identify bacterial genes that are induced during infection (26, 27). Such in vivo-induced (*ivi*) genes are expressed poorly on rich laboratory media (i.e., Luria broth [LB] or lactose MacConkey) but undergo elevated levels of expression in host tissues. Using this system, we previously identified over 100 different *Salmonella typhimurium* genes that are induced during infection of BALB/c mice and/or RAW 264.7 cultured murine macrophages (23). The functions of 25% of these *ivi* genes are unknown: they show no significant homology to entries in the DNA databases (8, 24). Many *ivi* genes reside in *Salmonella*-specific regions of atypical base composition that encode predicted adhesin and invasin-like functions required for full virulence. Several of these *Salmonella*-specific regions are inherited in a serovar-specific fashion (9).

Many virulence genes shown to be coordinately regulated in vitro are presumed to function at the same anatomical site during infection. For example, the production of cholera toxin in vitro is coordinately regulated by the same environmental (pH, temperature, and osmolarity) and genetic (e.g., ToxR) signals as production of the toxin-coregulated pilus, both of which function in the small intestine (28, 42). Thus, independent of the actual (often unknown) in vivo signals, classification of bacterial genes based on regulatory patterns in vitro may reflect coordinate expression at a given anatomical site as well as a possible functional relationship (i.e., in the same biochemical pathway or in a broader context, such as phagosome survival).

Here, we have implemented the *lac* fusion technology of the IVET approach to classify *ivi* genes isolated from different host tissues (spleen, liver, intestine) based on their coordinate expression patterns in response to environmental (pH, Mg^{2+}, and Fe^{2+}) and regulatory (PhoPQ) conditions known to control virulence gene expression in vitro (28–30). Low pH and low Mg^{2+} have been associated with *Salmonella*-containing vacuoles (15) and have been shown to be relevant signals for bacterial genes presumed to function in the macrophage (16, 39, 40). Low Mg^{2+} is the inducing signal for the activation of the PhoPQ regulatory system (16) which controls *Salmonella* virulence (18), including functions required for survival within (31) and during acidification of (2) the macrophage phagosome. Additionally, iron limitation is a well-characterized barrier to infection and thus may serve as an important environmental signal influencing bacterial gene expression in the animal (11, 28).

Here we demonstrate that *ivi* genes which are coordinately induced in response to low pH and low Mg^{2+} also displayed coordinate induction upon entry into cultured murine macrophages and cultured human epithelial cells. These data suggest this coordinately expressed class of *ivi* genes responds to general intracellular signals present during both early and late stages of infection.

MATERIALS AND METHODS

Media. Laboratory media used in these studies included LB (10) and MOPS [3-(*N*-morpholino)propanesulfonic acid] minimal medium (33) with 0.4% glycerol as a carbon source to avoid catabolite repression effects. When necessary, the pH was adjusted to pH 7.6, 7.0, 6.6, or 5.5. To buffer media to pH 5.5, MOPS was replaced with MES [2-(*N*-morpholino)ethanesulfonic acid] at the same concentration. MOPS medium contains 10 μM iron. Medium stocks were prepared iron or Mg^{2+} free when required, and $FeSO_4$ or $MgCl_2$ was added at the desired concentration. Unless specified otherwise, final concentrations of antibiotics (Sigma) in LB and minimal medium were as follows: ampicillin, 50 and 15 μg/ml; tetracycline, 20 and 10 μg/ml; and chloramphenicol, 20 and 5 μg/ml.

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TABLE 1. *S. typhimurium* strains used in this study.

Strain[a]	Genotype
MT1228	DUP3169 [Φ(*mgtA′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*mgtA*$^+$)]
MT1865	DUP3169 [Φ(*mgtA′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*mgtA*$^+$)] *phoP102*::Tn*10d*-Cm
MT1866	DUP3169 [Φ(*mgtA′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*mgtA*$^+$)] *phoQ24*
MT1695	DUP3170 [Φ(*mgtB′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*mgtB*$^+$)]
MT1696	DUP3170 [Φ(*mgtB′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*mgtB*$^+$)] *phoP102*::Tn*10d*-Cm
MT1697	DUP3170 [Φ(*mgtB′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*mgtB*$^+$)] *phoQ24*
MT1701	DUP3210 [Φ(*spvB′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*spvB*$^+$)]
MT1702	DUP3210 [Φ(*spvB′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*spvB*$^+$)] *phoP102*::Tn*10d*-Cm
MT1703	DUP3210 [Φ(*spvB′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*spvB*$^+$)] *phoQ24*
MT1704	DUP3188 [Φ(*iviVI-A′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*iviVI-A*$^+$)]
MT1705	DUP3188 [Φ(*iviVI-A′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*iviVI-A*$^+$)] *phoP102*::Tn*10d*-Cm
MT1706	DUP3188 [Φ(*iviVI-A′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*iviVI-A*$^+$)] *phoQ24*
MT1818	DUP3350 [Φ(*iviXVI′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*iviXVI*$^+$)]
MT1898	DUP3350 [Φ(*iviXVI′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*iviXVI*$^+$)] *phoP102*::Tn*10d*-Cm
MT1899	DUP3350 [Φ(*iviXVI′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*iviXVI*$^+$)] *phoQ24*
MT1371	DUP3193 [Φ(*phoP′-purA*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET1*(*phoP*$^+$)]
MT1681	DUP3193 [Φ(*phoP′-purA*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET1*(*phoP102*::Tn*10d*-Cm)]
MT1680	DUP3193 [Φ(*phoP′-purA*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET1*(*phoP*$^+$)] *phoQ24*
MT1698	DUP3465 [Φ(*pmrB′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*pmrB*$^+$)]
MT1699	DUP3465 [Φ(*pmrB′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*pmrB*$^+$)] *phoP102*::Tn*10d*-Cm
MT1700	DUP3465 [Φ(*pmrB′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*pmrB*$^+$)] *phoQ24*
MT1819	DUP3371 [Φ(*iviXVII′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*iviXVII*$^+$)]
MT1900	DUP3371 [Φ(*iviXVII′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*iviXVII*$^+$)] *phoP102*::Tn*10d*-Cm
MT1901	DUP3371 [Φ(*iviXVII′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*iviXVII*$^+$)] *phoQ24*
MT1772	DUP3142 [Φ(*fhuA′-purA*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET1*(*fhuA*$^+$)]
MT1833	DUP3127 [Φ(*cirA′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*cirA*$^+$)]
MT1801	DUP3231 [Φ(*entE′-cat*$^+$-*lacZ*$^+$*Y*$^+$)*pIVET8*(*entF*$^+$)]

[a] All derived from *S. typhimurium* ATCC 14028 (CDC 6516-60).

Bacterial strains and phage. All *S. typhimurium* strains used in this study were derived from strain ATCC 14028 (CDC 6516-60). The high-frequency generalized transducing bacteriophage P22 mutant HT 105/1, *int*-201 was used for all transductional crosses (36), and phage-free, phage-sensitive transductants were isolated as previously described (7). Strains used for PhoPQ regulation studies were constructed by transduction of the IVET-selected fusion into ATCC 14028 (wild type) and isogenic *phoPQ* derivatives MT1657 (*phoP102*::Tn*10d*-Cm [32]) and MT1658 (*phoQ24* [31]), kindly provided by Karl Klose (University of Texas, San Antonio).

Cell culture. The murine macrophage cell line RAW 264.7 and human epithelial cell lines HEp-2 (larynx carcinoma) and Henle-407 (embryonic small intestine) (ATCC TIB-71, CCL-23, and CCL-6, respectively) were obtained from the American Type Culture Collection, Rockville, Md., and maintained in minimum essential medium (MEM) supplemented with Earle's salts, L-glutamine, and 10% heat-inactivated fetal calf serum (FCS) (Life Technologies, Rockville, Md.). Cells were grown in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C in 75-cm^2 plastic flasks (Corning Glass Works, Corning, N.Y.). Cultured macrophages were harvested by scraping with a rubber policeman, or in the case of epithelial cells, by trypsinization using 0.25% trypsin–0.02% EDTA, and plated at a density of 2.5×10^5 to 5×10^5 cells/ml in 4 ml of culture medium in 35-mm-diameter, six-well dishes (Corning) and grown for 24 h to approximately 80 to 90% confluence (1×10^6 to 5×10^6 cells/well) (14).

Cultured macrophage and epithelial cell infections. *S. typhimurium* was grown overnight without shaking in LB containing ampicillin. Cultured cells were inoculated with *S. typhimurium* at a bacterium/host cell ratio of 10:1. Fifty microliters of an overnight culture of bacteria (5×10^7) was added to 5×10^6 cultured macrophages in six-well cell culture plates (Corning); 10^7 bacteria were added to 10^6 cultured epithelial cells. The bacteria were centrifuged onto cultured monolayers at $1{,}000 \times g$ for 10 min at room temperature, after which they were incubated at 37°C for 30 min in a 5% CO_2 incubator. The coculture was washed twice with cell culture medium and incubated for 2 h in the presence of 100 μg of gentamicin per ml to kill extracellular bacteria (17). Cells were washed twice with cell culture medium and incubated for the time specified in cell culture medium containing 10 μg of gentamicin per ml. The coculture was then washed three times with ice-cold 1× phosphate-buffered saline (PBS), the host cells were lysed with 1% Triton X-100, and the surviving intracellular bacteria were recovered in 1× PBS. β-Galactosidase assays were performed on the recovered bacterial cells, which were also plated for single colonies to determine bacterial cell number.

β-Galactosidase assays. β-Galactosidase activities were assayed by the method of Slauch and Silhavy (37). Unless otherwise specified, activities are given as 10^3 units per A_{600} unit per milliliter of cell suspension, where units are micromoles of *o*-nitrophenol (ONP) formed per minute ($n = 3$ trials, standard deviation $< 10\%$ of the mean).

Mg^{2+} and pH assays. Bacterial strains were used to inoculate 1 ml of minimal medium at the given pH (7.6, 6.6, or 5.5) and Mg^{2+} concentration (10, 1, 0.05, or 0 mM). When Mg^{2+} was omitted from the incubation medium, sufficient growth was obtained to reach a final optical density at 600 nm (OD_{600}) of at least 0.2 for the *phoP*$^+$ strains; *phoP* and *phoQ24* strains failed to grow under these conditions. The cultures were shaken at 37°C, and β-galactosidase assays were performed after 16 h. To assay cells grown in exponential phase, growth curves were determined for each environmental condition. Overnight cultures grown in the 12 assay conditions tested were diluted (typically 12- to 30-fold) to an OD_{600} of 0.1 in the same medium (cells were not tested in the absence of Mg^{2+} since the strains do not grow well in this medium after dilution). The subcultures were grown at 37°C and assayed at an OD_{600} of 0.4 to 0.6.

Iron assays. Bacterial strains were used to inoculate 1 ml of MOPS minimal medium at the designated pH and Mg^{2+} concentration with the addition of the given concentration of $FeSO_4$. Medium conditions without iron added also contained the iron-chelating agent 2,2′-dipyridyl (0.03 mM). The cultures were shaken at 37°C, and β-galactosidase assays were performed after 16 h of incubation.

Cell culture assays. Bacterial cells were recovered from infected cultured macrophages or epithelial cells lysed with 1% Triton X-100 in 1× PBS. β-Galactosidase activities typically were assayed on 0.5 ml of the 1 ml coculture lysate. The activity was determined as described by Slauch and Silhavy (37), with the modification that the number of bacteria was determined by direct colony count; units were expressed as micromoles of ONP formed per minute per CFU. For comparison, bacteria were also grown in 1 ml of LB and cell culture medium containing 10% FCS without shaking; assays performed on the recovered bacteria (typically 0.1 ml) were determined as in the cell culture assay. β-Galactosidase activities for experiments were determined in triplicate on at least two different days.

RESULTS

pH and Mg^{2+} regulation of *ivi* genes. We have tested the effects of the environmental signals pH and Mg^{2+} on the expression patterns of 74 IVET-selected bacterial gene fusions recovered from infected BALB/c mice (intestine, spleen, and liver) and/or from cultured RAW 264.7 murine macrophages (23). These environmental signals have been associated with

Salmonella-containing vacuoles (15) and are presumed to be relevant signals in the macrophage environment (16, 39, 40). The strains used in this study are shown in Table 1, and the environmental and genetic parameters governing their expression are summarized in Table 2. The strains presented in Table 2 exhibited a greater than twofold response to the signals tested. Figure 1 shows the responses of eight *ivi* fusions following 16 h of growth under a wide variety of Mg^{2+} and pH conditions. Fusions in strains that are wild type with respect to *phoPQ* were also assayed in exponential phase; both the actual values of β-galactosidase activity and the expression pattern of each fusion were similar to those observed after 16 h of growth (data not shown).

Figure 1 shows a set of eight *ivi* fusions that respond to pH and Mg^{2+} in the media; each fusion displays a characteristic response to these signals. Examples include five known *ivi* genes and three unknown *ivi* genes, some of which were shown previously to respond to these environmental conditions (referenced in Table 2). Known genes include *mgtA* and *mgtB* which encode two high-affinity Mg^{2+} transport systems; *mgtB*, part of an operon with a gene (*mgtC*) residing on *Salmonella* pathogenicity island 3 and required for intramacrophage survival (4); *spvB*, which resides on the *Salmonella* virulence plasmid and facilitates growth at systemic sites of infection (19); and *phoP* and *pmrAB*, which encode two-component regulatory systems that affect the expression of *Salmonella* virulence genes (18). Unknown genes include *iviVI-A* (23), which encodes a predicted product that shows extensive sequence similarity to enterotoxigenic *Escherichia coli* Tia, an outer membrane protein involved in attachment to and invasion of gut epithelial cells (14). We have mapped *iviXVI* to between min 30 and 37 on the *Salmonella* chromosome (35), and its predicted amino acid sequence (using 262 bp from the fusion joint) shows similarity (57 to 62% identity) to several aldehyde dehydrogenases of *E. coli*. We have recently identified *iviXVII* as a member of the *pdu* operon (9), involved in vitamin B_{12}-dependent 1,2-propanediol utilization (34).

Figure 1A shows that seven of eight *ivi* fusions recovered from the spleen and/or cultured macrophages are induced by both low pH and low Mg^{2+}, an environment likely to be found within *Salmonella*-containing vacuoles (15). However, although *iviXVII* (*pdu*) was also recovered from the spleen, Fig. 1B shows that it exhibits a reciprocal pattern of expression with regard to pH and Mg^{2+}: it is induced when grown under high-Mg^{2+} and mild alkaline conditions but repressed when grown under low-pH and low-Mg^{2+} conditions. Thus, although *pdu* is capable of responding to signals in a different fashion than the other *ivi* fusions, the data in Fig. 1B and 2 show that under conditions presumed to exist in vacuolar environments, *pdu* expression may have been sufficient for IVET selection in the spleen.

Regulation of *ivi* genes by PhoPQ. To determine if the environmental regulation was mediated through the PhoPQ regulatory system, the expression patterns of *ivi* fusions found in the wild type were compared to those observed in a $PhoP^-$ (*phoP102*::Tn*10d*-Cm) genetic background. Figure 1A (second column) shows that all of the eight *ivi* genes that respond to both pH and Mg^{2+} are also PhoP-regulated genes (the responses are summarized in Table 2). Seven of these (*mgtA*, *mgtB*, *spvB*, *phoP*, *pmrB*, *iviVI-A,* and *iviXVI*) are PhoP-activated genes (*pags* [16, 21, 23, 38–40]), since their expression is dependent on the presence of a functional PhoP regulatory protein. Note that there is suboptimal expression of *pmrB* in the absence of PhoP under several environmental conditions (e.g., pH 6.6 and 1 mM Mg^{2+}). Conversely, Fig. 1B shows that the expression levels of *iviXVII* (*pdu*) are increased under

TABLE 2. Environmental and genetic regulation of *S. typhimurium ivi* genes

Strain	Gene	Function	Environmental signal(s)	PhoPQ regulation	Parameters	Reference(s)[a]
MT1371	*phoP*	Virulence regulator	pH + Mg^{2+}	*pag*	i.g., i.p./spleen[b]	16, 23, 38, 40, this work
MT1698	*pmrB*	Polymyxin resistance	pH + Mg^{2+}	*pag*	Cultured macrophage	21, 23, 39, this work
MT1701	*spvB*	Plasmid virulence	pH + Mg^{2+}	*pag*	i.p./spleen	13, 23, this work
MT1228	*mgtA*	Mg^{2+} transport	pH + Mg^{2+}	*pag*	i.p./spleen; cultured macrophage	16, 23, 39, 40, this work
MT1695	*mgtB*	Mg^{2+} transport	pH + Mg^{2+}	*pag*	i.p./spleen	15, 16, 23, 40, 41, this work
MT1704	*iviVI-A*	Adhesin-like	pH + Mg^{2+}	*pag*	i.p./spleen	23, this work
MT1818	*iviXVI*	Aldehyde dehydrogenase-like	pH + Mg^{2+}	*pag*	i.p./spleen	This work
MT1819	*iviXVII (pdu)*	1,2-Propanediol utilization	pH + Mg^{2+}, iron O_2, propanediol	*pag*	i.p./spleen	1, 5, this work
MT1772	*fhuA*	Iron transport	Iron	*None*	i.g./small intestine	23, 41, this work
MT1833	*cirA*	Colicin 1 receptor	Iron	*None*	Cultured macrophage	23, this work
MT1801	*entF*	Enterobactin synthesis	Iron	*None*	Cultured macrophage	23, this work

[a] Listed are references relevant to environmental signals that affect *ivi* gene expression in *S. typhimurium*.
[b] Route of delivery (intragastric [i.g.] or intraperitoneal [i.p.])/host tissue from which bacteria were recovered.

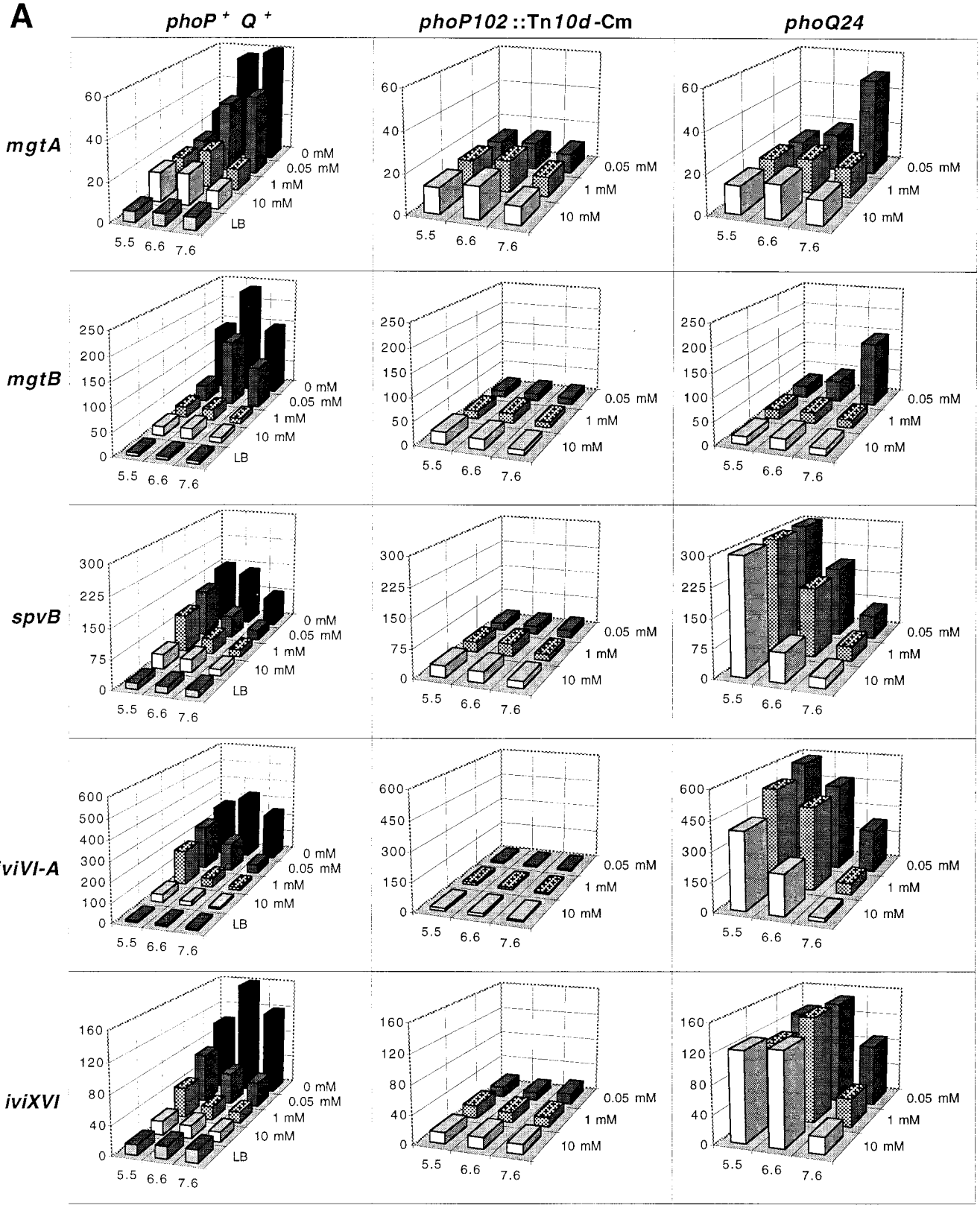
A
phoP+ Q+
phoP102::Tn10d-Cm
phoQ24
mgtA
mgtB
spvB
iviVI-A
iviXVI
0 mM
0.05 mM
1 mM
10 mM
LB
5.5
6.6
7.6

FIG. 1.

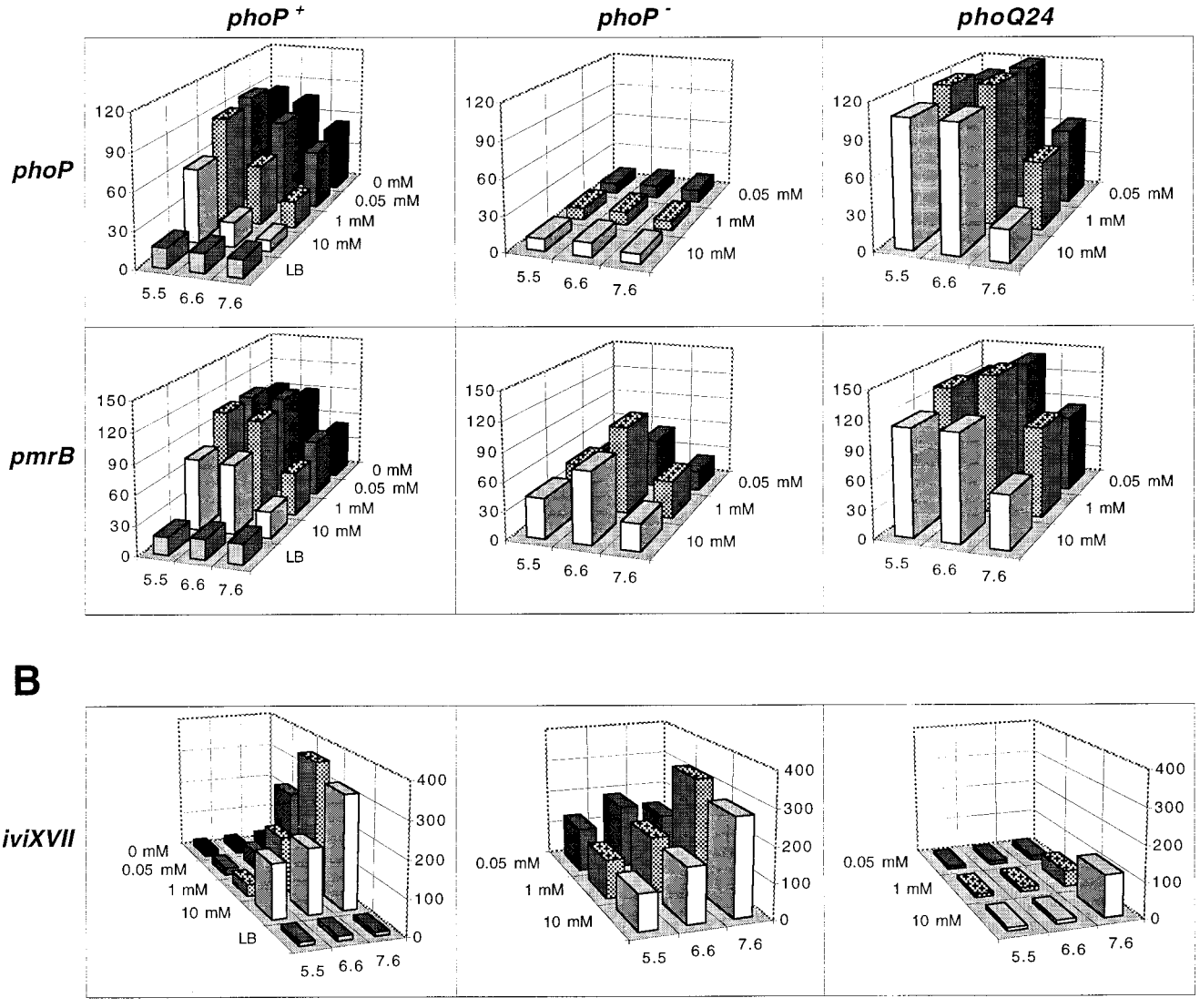

FIG. 1. Expression profiles of *ivi* fusions in response to pH and Mg^{2+} concentration. pH values are represented on the *x* axis, β-galactosidase activities [(micromoles of ONP formed per minute per A_{600} unit per milliliter of cell suspension] [10^3]) are plotted on the *y* axis, and Mg^{2+} concentrations are plotted on the *z* axis. Values represent the averages of three independent cultures with a standard deviation of <10% of the mean. (A) Seven PhoP-activated *ivi* fusions. (B) PhoP-repressed fusion *iviXVII* (*pdu*). In panels A and B, the columns represent, from left to right, expression profiles in a $PhoP^+$ background, in a $PhoP^-$ (*phoP102*::Tn*10d*-Cm) background, and in a *phoQ24* background. A very similar induction profile was obtained for exponentially growing cells of the wild type under all 15 conditions tested (data not shown). The pH of each wild-type culture was quantitated under the 15 conditions tested before and after incubation. After 16 h of growth, the pH in MOPS minimal medium typically decreased 1 to 1.8 pH units; the pH in MOPS-buffered LB increased 1.0 to 1.5 pH units. The pH of exponentially grown cultures in MOPS minimal medium typically decreased 0.4 to 0.6 pH units in MOPS minimal medium or remained within 0.4 pH units in MOPS-buffered LB.

several conditions in the absence of PhoP, and thus *iviXVII* is defined as a PhoP-repressed gene (*prg*).

The conformation of the PhoQ sensor protein has been shown to respond to levels of Mg^{2+} in the medium (16). The *phoQ24* mutation renders the PhoQ protein less sensitive to its known inhibitory signal, Mg^{2+} (16), resulting in enhanced phosphorylation of PhoP (20). Such enhanced PhoP phosphorylation causes increased expression of *pags* and reduced expression of *prgs* (31). As expected, the effect of *phoQ24* is to amplify the expression of the PhoP-activated genes *spvB*, *phoP*, *pmrB*, *iviVI-A*, and *iviXVI* compared to that found in the wild type over a wide range of conditions (Fig. 1A, third column). Figure 1B shows that *phoQ24* abolishes the expression of *iviXVII* (*pdu*) under most conditions tested, as expected for a *prg*.

Iron regulation of *ivi* genes. The 74 IVET-selected fusions that were tested for their response to Mg^{2+} and pH were also screened for their response to iron. Three *ivi* fusions, *fhuA*, *cirA*, and *entF*, are induced 5-, 8-, and 22-fold, respectively, by iron limitation (no iron added, 0.03 mM 2,2′-dipyridyl) compared to growth in iron excess (0.01 mM iron), as shown previously for *E. coli* (reference 11 and data not shown). Each of these *ivi* genes is presumed to play a role in iron transport: *fhuA* encodes a siderophore uptake system; *cirA* encodes the colicin 1 receptor, which also shows extensive homology to siderophore uptake systems; and *entF* encodes enterobactin synthetase component F (11). Conversely, *iviXVII* (*pdu*) expression increased 13-fold when cells were grown under high versus low iron conditions in minimal medium but not in the

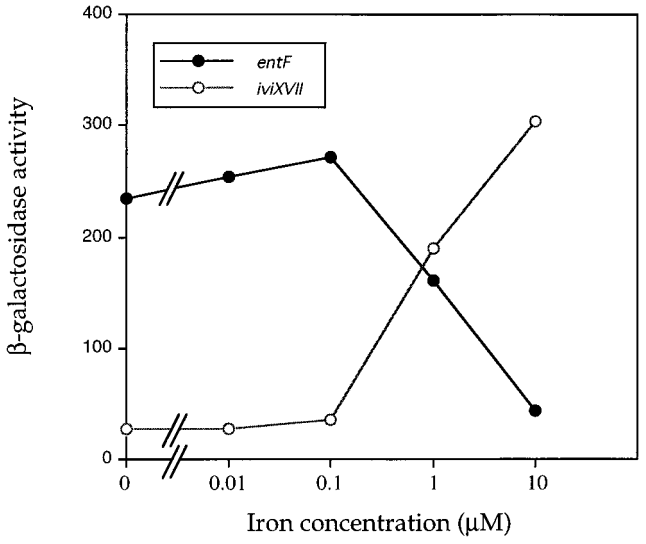

FIG. 2. *iviXVII* (*pdu*) and *entF* expression as a function of iron concentration in the medium. Cultures were grown overnight in iron-free MOPS minimal medium at pH 7.0. The cultures were diluted to OD_{600} of 0.1 in the same medium grown to an OD_{600} of 0.4 (mid-log phase). $FeSO_4$ was then added at the given concentration, and β-galactosidase activities were determined after 5 h. Values represent the averages of three independent cultures with a standard deviation of <10% of the mean.

high-iron conditions present in LB (10 μM [reference 17 and data not shown]). Thus, all iron-responsive *ivi* fusions are repressed when grown in LB but must be expressed in the animal in response to the IVET selection.

To resolve the apparent paradox that reciprocally regulated fusions (with respect to iron) are repressed in LB and expressed in the animal (and in cultured mammalian cells [see below]), the effect of iron concentration on *entF* and *iviXVII* expression was quantitated. Figure 2 shows that in 1 μM iron (the concentration indirectly estimated for *Salmonella*-containing vacuoles [15]), both *entF* and *iviXVII* are expressed at high levels, presumably sufficient for both to respond to the IVET selection.

Table 3 shows the effects of *phoPQ*, medium composition (pH and Mg^{2+}), and iron on expression of *iviXVII* (*pdu*) in vitro. The data indicate that the ability of *iviXVII* to respond to high iron is dependent on the absence of the PhoP regulatory protein. This can be achieved by either growth condition or mutation. For example, *iviXVII* is induced by iron under growth conditions where PhoP is not expressed (pH 7.0 to 7.6 and 0.5 to 10 mM Mg^{2+} [Fig. 1B]) or in a $PhoP^-$ background.

TABLE 3. Effects of *phoPQ*, medium composition, and iron on expression of a *iviXVII*::*lac* (*pdu*) fusion

iviXVII::*lac* derivative	β-Galactosidase activity (U)[a]					
	pH 5.5, 0.05 mM Mg^{2+}		pH 7.0, 0.05 mM Mg^{2+}		pH 7.6, 10 mM Mg^{2+}	
	−Iron	+Iron	−Iron	+Iron	−Iron	+Iron
phoP$^+$	22	25	27	288	27	322
phoP102::Tn*10d*-Cm	13	216	15	361	13	344
phoQ24	11	10	12	15	13	247

[a] Determined in cultures grown for 16 h in MOPS minimal medium at the given pH and Mg^{2+} concentration in the presence or absence of 50 μM iron. Medium without iron added also contained the iron-chelating agent 2,2′-dipyridyl (0.03 mM). Data represent averages of three independent cultures with a standard deviation of <10% of the mean.

Intracellular expression of *ivi* genes. The eight *ivi* genes that respond to pH, Mg^{2+}, and PhoP in vitro were tested for expression upon entry into and growth within three cultured host cell lines: RAW 264.7 (murine macrophage) and HEp-2 and Henle-407 (human epithelium). Preselected Lac^- and Lac^+ strains were obtained from the initial nonselected pool of integrated IVET fusions. Figure 3 indicates that these strains showed no significant intracellular induction relative to the PhoP-regulated *ivi* genes which were induced at 4 h following infection of all three cell lines. While each *ivi* fusion has a characteristic level of intracellular induction, that level is relatively consistent within all three cell lines tested, as shown previously for *spvB* in several cell lines (13).

Effects of PhoPQ on intracellular *ivi* gene expression. To determine whether the regulatory behavior of *ivi* genes mediated by PhoPQ in vitro mimics what is observed intracellularly, we assayed the effect of PhoPQ on strains containing either PhoP-activated or PhoP-repressed *ivi* genes in cultured macrophages. Figure 4 shows that introduction of a PhoP mutation into either *pag* strain (*iviVI-A* and *spvB*), resulted in reduced intracellular expression in cultured RAW 264.7 macrophages, indicating that PhoP is a major regulatory protein involved in the expression of these *ivi* genes in vitro and in cultured cells. In contrast, the intracellular expression of the PhoP-repressed gene *iviXVII* (*pdu*) was not enhanced after introduction of a PhoP mutation. This finding suggests that intracellular PhoP is not phosphorylated sufficiently to repress the low-level intracellular expression of *iviXVII* and/or other factors contribute to *iviXVII* expression 4 h postinfection. Additionally, *phoP* and *iviXVII* show significant levels of expression under some common conditions in vitro (Fig. 1) and during growth within cultured mammalian cells (Fig. 3). Taken together, these data suggest that although *pags* and *prgs* are capable of responding differently to a similar set of signals, this does not preclude their concomitant expression within the same cell type (see Discussion).

DISCUSSION

Here we have grouped several *ivi* genes based on their coordinate behavior in response to environmental and genetic signals known to control virulence gene expression in vitro. Such in vitro expression was shown to parallel their intracellular induction both in cultured murine macrophages and in human epithelial cells. Moreover, the *ivi* fusions are each induced to similar levels in both cell types, suggesting that they respond to environmental and genetic signals that are present at both early and late stages of infection. These coordinately expressed *ivi* genes reside in three different types of DNA sequences, including native chromosomal sequence (*mgtA*), plasmid sequence (*spvB*), and acquired sequence of atypical base composition (*iviVI-A*). Thus, acquired sequences either have evolved to respond to the regulatory circuitry of the recipient *Salmonella* genome or were responsive to similar regulatory controls in the donor organism.

All known *ivi* genes analyzed in this study have been shown to be required for, or have been implicated in, virulence (9, 23). Additionally, two genes, *iviVI-A* and *iviXVII* (*pdu*), reside in regions of atypical base composition whose removal confers virulence defects (9). There is a strong correlation between the expression of *ivi* genes in response to a given set of environmental and genetic signals in vitro and their intracellular expression in mammalian cells. All *ivi* fusions that were induced by low pH and low Mg^{2+} were activated by PhoPQ. These

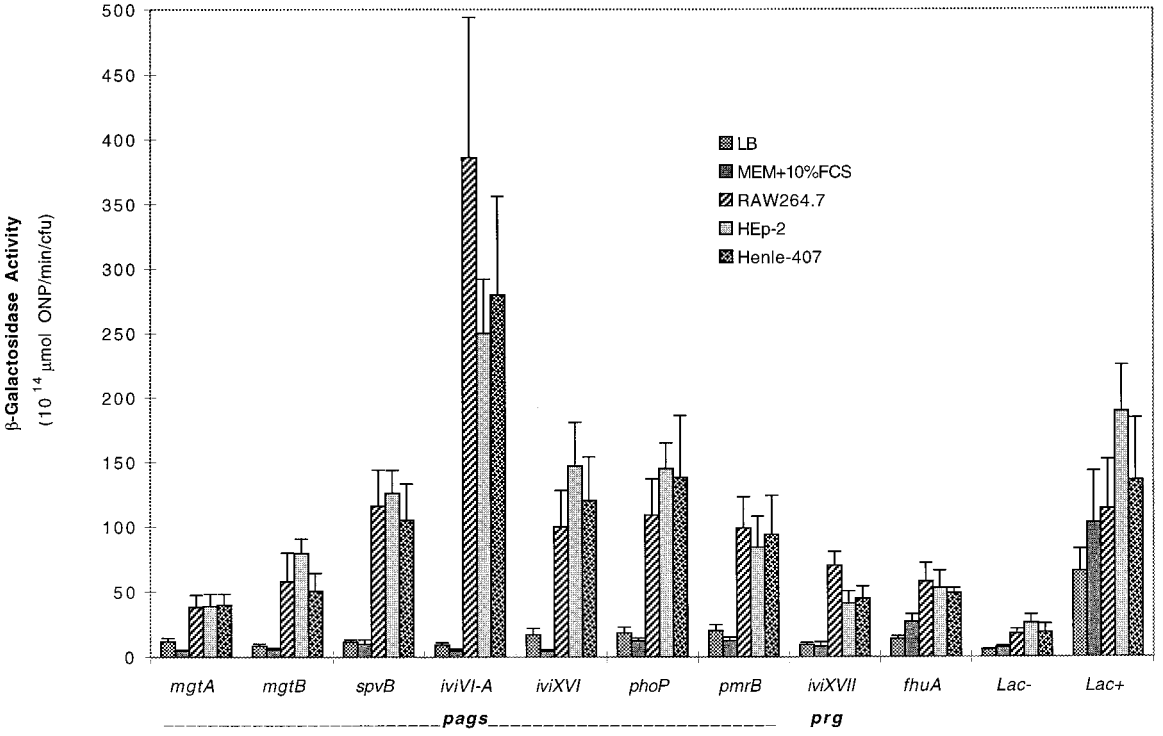

FIG. 3. Intracellular expression of *ivi* genes in cultured macrophages and cultured epithelial cells. Individual *ivi* fusions were incubated with cultured RAW 264.7 murine macrophages and cultured human epithelial cell lines HEp-2 (larynx carcinoma) and Henle-407 (embryonic small intestine). The coculture was incubated for 30 min, washed, treated with gentamicin to kill extracellular bacteria, washed, and incubated for 4 h. The cultured mammalian cells were lysed with Triton X-100, and β-galactosidase assays were performed on the recovered intracellular bacteria. For comparison, β-galactosidase assays were also performed on individual *ivi* fusions grown for 4 h in LB and in MEM supplemented with 10% FCS. Preselected Lac$^-$ and Lac$^+$ strains were obtained from the initial nonselected pool of integrated IVET fusions. Error bars represent 1 standard deviation of the measured value.

genes were also induced upon entry into and growth within mammalian cells; moreover, *spvB* and *iviVI-A* were also shown to be activated by PhoPQ in cultured macrophages. Not all *pags* require PhoP for their induction under all environmental conditions. Notably, *pmrB* showed significant expression in the absence of PhoP (e.g., pH 6.6 and 1 mM Mg^{2+}), which may be important under conditions found in the host. Coordinate intracellular expression of genes that respond similarly in vitro (e.g., low pH and low Mg^{2+}) may reflect their response to similar vacuolar environments in these cell types. Defining the bacterial genes that are expressed in *Salmonella*-containing vacuoles in macrophages and/or epithelial cells will identify both general and specific functions that may be required for survival in these intracellular microenvironments as has been shown recently (6).

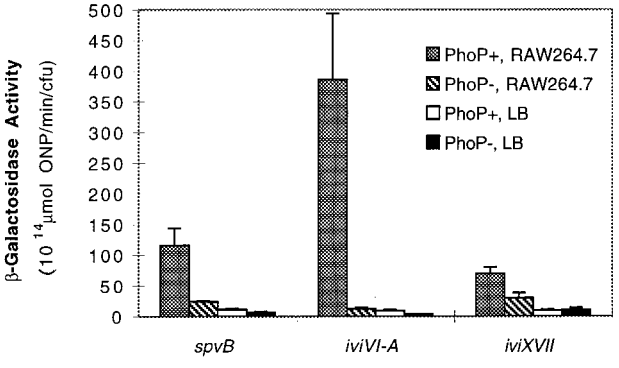

FIG. 4. Intracellular expression of *ivi* genes in PhoP$^+$ and PhoP$^-$ (*phoP102*::Tn*10d*-Cm) backgrounds in cultured macrophages. Individual *ivi* fusions were incubated with cultured RAW 264.7 murine macrophages. The coculture was incubated for 30 min, washed, treated with gentamicin to kill extracellular bacteria, washed, and incubated for 4 h. The cultured macrophages were lysed with Triton X-100. β-Galactosidase assays were performed on the recovered intracellular bacteria and also on individual *ivi* fusions grown for 4 h in LB. Error bars represent 1 standard deviation of the measured value.

It is not implied that *ivi* genes used in this study are expressed only in the cell types tested or that other *ivi* genes are not expressed in the same cell types or subcellular compartments. For example, previously we have shown that *phoP* is induced after both intragastric and intraperitoneal inoculation (23), indicating that *phoP* is expressed at early and late stages of infection presumably in several cell types. Indeed, it has been recently shown that PhoPQ is a component of the acid tolerance response (3), which is presumed to contribute to bacterial survival at many anatomical sites. Additionally, most *ivi* genes isolated from the same infected tissue (e.g., spleen) do not respond to any of the signals tested here (e.g., iron, Mg^{2+}, pH, and PhoP), suggesting that other signals may govern their expression in these tissues.

PhoP-activated and PhoP-repressed genes are capable of responding to the same set of signals in diverse and robust fashion. However, both classes show significant levels of expression under the same conditions in vitro (Fig. 1) and during growth within cultured mammalian cells (Fig. 3). Thus, *pags* and *prgs* may be induced within the same cell type and subcellular compartment (e.g., macrophage phagosome). The low level induction of *iviXVII* (*pdu*) observed within cultured macrophages may make a significant contribution to the intracel-

lular fitness of the bacterium within the host. Indeed, the extent to which an individual gene is induced relative to another gene is not necessarily an indication of how important a particular gene is to a particular downstream event; it is simply a measurable factor. Correspondingly, removal of the *pdu* region confers a defect in systemic survival (9).

The expression pattern of *spvB* presented here differs from results of previous studies using alternatively constructed fusions. SpvB translational fusions from a low-copy-number plasmid containing *S. dublin* sequences have been reported to be induced in a PhoP-independent manner (12); moreover, the fusion protein and native mRNA levels are induced in stationary phase (12, 25). The *spvB* transcriptional fusion in this study resides at its native site on the *S. typhimurium* virulence plasmid, is expressed in LB at very low levels, is activated by PhoP, and is subject to coordinate environmental and genetic regulation with other PhoP-activated genes both in vitro and in cultured mammalian cells. One explanation for this disparity may lie in the structure of the *spvB ivi* fusion construct. Although the integrated *spvB*::*lac* fusion contains all *spv* wild-type sequences and their native control regions, two types of messages are produced since the promoter region is duplicated in this construct (covering 3 kb of sequence upstream of the *spvB* fusion join). Thus, the *spvB*::*lac* fusion-bearing message does not contain sequences that may be relevant to production and/or stability of full-length message in stationary phase, allowing the observation of subtle but significant aspects of *spv* regulation whose biological role is yet to be determined.

Given the intracellular induction of a subset of *ivi* genes, we now can ask how these genes contribute to bacterial survival during infection. One intracellularly expressed fusion, *iviVI-A*, resembles an enterotoxigenic *E. coli* adhesin and the Opa adherence and invasion proteins of *Neisseria gonorrhoeae*. The intracellular expression of an adhesin/invasin-like protein may seem puzzling. However, bacterial proteins often play roles other than those for which they were first defined. Indeed, the Opa adherence proteins have an additional function once the gonococci are intracellular: these outer membrane proteins bind host pyruvate kinase at the bacterial surface, leading to an environment encompassing the bacterium that is rich in pyruvate, which is one of few known carbon sources utilized by *N. gonorrhoeae* in vitro. (43). Thus, the adhesin-like IviVI-A protein may contribute other capabilities to *S. typhimurium* within host vacuoles.

Determination of the genetic and environmental factors that regulate *ivi* expression and the host site(s) in which they are expressed provides clues to both the intracellular environment and possible functions of *ivi* genes at these specific host sites. This complex and overlapping regulatory circuitry offers a pathogen tremendous flexibility to express different sets of genes at various sites. The functions of some of these genes may change dependent on the context of the animal (chicken versus cow), tissue (intestine versus spleen), cell type (macrophage versus hepatocyte), or subcellular compartment (phagosome versus phagolysosome).

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